

Aspirin and Salicylates Inhibit the IL-4- and IL-13-Induced Activation of STAT6¹

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Allergic diseases, including asthma, represent a major threat to human health. Over the three last decades, their incidence has risen in western countries. Aspirin treatment has been shown to improve allergic diseases, especially asthma, and the decreased use of aspirin has been hypothesized to contribute to the increase in childhood asthma. Because salicylate compounds suppress a number of enzymatic activities, and signaling through IL-4R participates in the development of allergic responses, we tested the effect of salicylates on IL-4 signal transduction. We found that treatment of cell lines and primary cells with aspirin and salicylates, but not acetaminophen, inhibited the activation of STAT6 by IL-4 and IL-13. This effect correlated with the inhibition of IL-4-induced CD23 expression. Although salicylates inhibited the *in vivo* activation of Janus kinases, their kinase activity was not affected *in vitro* by salicylates, suggesting that other kinases were involved in IL-4-induced STAT6 activation. Furthermore, we found that an Src kinase was involved in STAT6 activation because 1) Src kinase activity was induced by IL-4, 2) Src kinase activity, but not Janus kinase, was inhibited by salicylates *in vitro*, 3) cells expressing viral Src had constitutive STAT6 phosphorylation, and 4) cells lacking Src showed low STAT6 phosphorylation in response to IL-4. Because STAT6 activation by IL-4 and IL-13 participates in the development of allergic diseases, our results provide a mechanism to explain the beneficial effects of aspirin and salicylate treatment of these diseases. *The Journal of Immunology*, 2002, 168: 1428–1434.

Human allergic diseases represent a serious threat to the health and well being of individuals (1, 2). Over the three last decades, their incidence has risen at an alarming rate in western countries; currently, ~30% of western populations suffer from allergies, including asthma, rhinitis, and atopic eczema (1, 2). A simple explanation for this phenomenon has not been found, and multiple potential causative factors have been proposed (1–9). They include exposure to indoor and outdoor air pollution, extensive vaccination programs, reduction in exposure to childhood infections, responses to certain early childhood viral infections, and responses to medications (1–9). Among medications, it has been hypothesized that the decreased use of aspirin to treat febrile respiratory infections may be a contributing factor to the increase in childhood asthma observed in the United States (5).

Aspirin (acetyl salicylic acid (ASA)³) has been one of the most widely used drugs in history. Since 1899, it has been used as an analgesic, an antipyretic, and an anti-inflammatory agent (10). Over the years, aspirin has been substituted by other agents due its side effects, especially in children (5, 10, 11). However, there are studies that support a beneficial effect of aspirin in asthma patients. Several groups showed that salicylates could protect against aller-

gic responses. Aspirin (12), lysine acetylsalicylate (13, 14), and sodium salicylate (NaSal; Ref. 14) were shown to protect the early and late asthmatic response to allergens. Salicylate pretreatment also attenuates the intensity of bronchial and nasal symptoms in aspirin-induced asthma (AIA; Ref. 15), and treatment of aspirin-sensitive patients with aspirin after desensitization results in clinical improvement of inflammatory respiratory disease (16).

The many effects of aspirin are believed to be mediated by the inhibition of cyclooxygenase (COX)-1 and COX-2 enzymes, thereby blocking the production of PG (10, 17). However, the concentrations of aspirin required to obtain a beneficial effect in asthma are higher than those required to inhibit COX (16). Furthermore, NaSal, shown to have a protective effect in asthma, has little effect on COX activity. These findings raise the possibility that the beneficial effect of salicylates on allergic processes is independent of COX inhibition. Recent observations indicated that salicylates can target a wider variety of enzymes. They can regulate the activation and inhibition of several kinases such as p38 mitogen-activated protein kinase (18) and I κ B kinase complex (19), therefore inhibiting the activation of NF- κ B (20). Recently, it was shown that aspirin can inhibit IL-4 gene transcription in CD4⁺ human T cells by an unknown mechanism (21).

IL-4 is a cytokine that participates in the immune response against parasitic infections (22) and in the development of allergic diseases (23–26). Its effects are mediated by a cell surface receptor expressed in most cell types. This receptor consists of two subunits, the IL-4R α -chain (IL-4R α) and the common γ -chain (γ_c) (27). IL-4R α binds IL-4 with high affinity and specifies the signals transmitted to the interior of the cell. The γ_c is shared by several cytokine receptors, including the IL-2R. In some cases, the γ_c can be substituted by the IL-13R $\alpha 1$ that, along with the IL-4R α , forms the IL-4R type II (28). Interestingly, the IL-4R α is also a member of the IL-13R complex (28), which explains why these two cytokines share many biological functions. At the intracellular level, signaling by IL-4 and IL-13 induces the activation of the transcription factor STAT6 via the Janus kinase (JAK) kinases (28–30). It has clearly been shown that the evolution of symptoms in the

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³ Abbreviations used in this paper: ASA, acetyl salicylic acid; COX, cyclooxygenase; AIA, aspirin-induced asthma; NaSal, sodium salicylate; γ_c , common γ -chain; IL-4R α , IL-4R α -chain; JAK, Janus kinase; v-Src, viral Src.

OVA-induced murine model of asthma is dependent on the development of Th2 cells and the production of the Th2 cytokines IL-4 (23–26) and IL-13 (31, 32). Furthermore, STAT6 activation is critical for efficient Th2 development in response to protein Ags and contributes to the asthma responses (33).

Because salicylates can regulate a number of enzymatic activities, we investigated the effect of salicylates on IL-4 and IL-13 signaling. We found that aspirin and NaSal inhibited the activation of STAT6 by a mechanism that likely involves the tyrosine kinase Src. The inhibition of STAT6 activation by salicylates may explain their beneficial effect on the treatment of allergic diseases.

Materials and Methods

Cells and reagents

NIH3T3-expressing viral Src (v-Src) were obtained from Dr. J. Moscat (Centro de Biología Molecular, Cantoblanco, Spain). Wild-type and Src knockout embryonic fibroblasts were obtained from Dr. X. Zhan (Holland Laboratory, American Red Cross, Rockville, MD) and have been previously described (34). Aspirin, NaSal, acetaminophenol, cycloheximide, and Na₃VO₄ were purchased from Sigma-Aldrich (St. Louis, MO), and MG132, lactacystin, and herbimycin A were obtained from Biomol (Plymouth Meeting, PA). Anti-JAK1 and -JAK3 were purchased from Upstate Biotechnology (Lake Placid, NY), RC20 anti-phosphotyrosine Ab was obtained from BD Transduction Laboratories (Lexington, Kentucky), and anti-STAT6, anti-phosphorylated STAT6, and src2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD23 Ab was a kind gift from Dr. J. M. Bergua (Hematology Department, Hospital San Pedro de Alcantara, Caceres, Spain). All cytokines were from R&D Systems (Minneapolis, MN).

EMSA

After culture, cells were washed and resuspended in lysis buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 10% glycerol, 200 mM NaCl, 1 mM DTT, 5 mM NaF, 0.1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture) for 5 min on ice. Cellular extracts were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 1 μg of protein was incubated with 1 ng of ³²P-labeled oligonucleotide in reaction buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 6% glycerol, and 0.1 mg/ml poly(dIdC)) for 20 min at room temperature. To determine STAT6 DNA-binding activity, we used the IFN-γ activation site sequence in the C_ε promoter (5'-CACTTCCCAAGAACAGA-3'). Polyacrylamide gels (4.5%) containing 0.22× Tris borate-EDTA were pre-run for 1 h at 200 V. After loading the samples, gels were run at 200 V for ~3 h. Afterward, gels were dried and exposed to film.

Immunoprecipitation and immunoblotting

After stimulation, cell pellets were lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor mixture) and clarified. To perform precipitations, the soluble fraction was immuno-

precipitated with the indicated Ab followed by incubation with protein G-agarose. The washed precipitates were separated on a 7.5% SDS-polyacrylamide gel before transfer to a polyvinylidene difluoride membrane. Membranes were then probed with the indicated Ab. The bound Ab was detected using ECL (Amersham, Arlington Heights, IL).

Kinase assays

JAK1, JAK3, and Src kinases were precipitated as described above. Precipitates were washed in kinase buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 50 μM Na₃VO₄, and protease inhibitors) and incubated in the presence of mentioned inhibitors. The enzymatic reaction was initiated with the addition of [³²P]ATP (Amersham). Samples were separated on polyacrylamide gels, dried, and exposed to film.

Analysis of CD23 expression

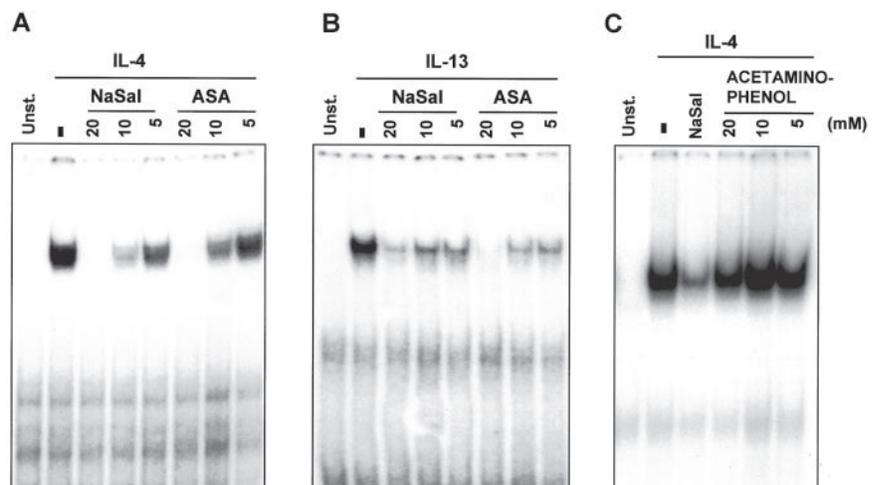
CD23 expression on monocytic cells was analyzed as previously described with little modification (35). PBMC were cultured for 30 h in the presence of indicated compounds. The cells were then stained with FITC-conjugated anti-human CD23 Ab (Immunotech, Marseille, France) and analyzed by flow cytometer (FACScan, BD Biosciences, Mountain View, CA). Viable monocytic cells were selected using forward-scatter and side-scatter parameters.

Results

Salicylates inhibit STAT6 activation

In experimental asthma, IL-4 and IL-13 have been shown to regulate a number of asthma symptoms (23–26, 31–33), likely mediated through STAT6 (33). Because salicylates also ameliorate asthma symptoms (12–16), we set out to test whether salicylates had an effect on IL-4- or IL-13-activated signal transduction. We first analyzed the effect of NaSal and ASA on the activation of STAT6 (Fig. 1). Pretreatment of the murine B cell lymphoma M12 with either NaSal or ASA blocked the ability of IL-4 to induce the DNA-binding activity of STAT6 in a concentration-dependent manner (Fig. 1A). A concentration of 20 mM completely blocked the activation of STAT6, whereas 5 mM inhibited STAT6 activation by >60%. Because ASA suppresses COX-1 and -2 activity and NaSal does not (16), these results suggest that inhibition of STAT6 activation by salicylates is not via suppression of COX activity. This inhibition was observed in all cell lines tested, including 32D, FDCP-1, M12, A1.1, U937, CH-31, and Wehi-231 (Figs. 1–3 and J. Zamorano, unpublished observations). The effect of salicylates in IL-4 signaling was not due to inhibition of IL-4 binding to its receptor because incubation of 32D cells with 20 mM NaSal did not affect the binding of IL-4 to its receptor (J. Zamorano, unpublished observations). We found similar results for IL-13 signaling; treatment of cells with NaSal and ASA also

FIGURE 1. Salicylates, but not acetaminophenol, inhibit STAT6 activation. *A*, M12 cells were cultured with the indicated concentrations of NaSal and ASA for 1 h before stimulation with IL-4 (10 ng/ml) for 30 min. STAT6 DNA-binding activity in cell extracts was analyzed by EMSA using the IFN-γ activation site sequence contained in the C_ε promoter. *B*, U937 cells were cultured with NaSal or ASA for 1 h, and then stimulated for 30 min with IL-13 (400 ng/ml). STAT6 activation was then analyzed by EMSA. *C*, M12 cells were cultured with nothing, NaSal (20 mM), or the indicated concentrations of acetaminophenol. Then, IL-4 was added, and STAT6 binding to DNA was analyzed by EMSA.



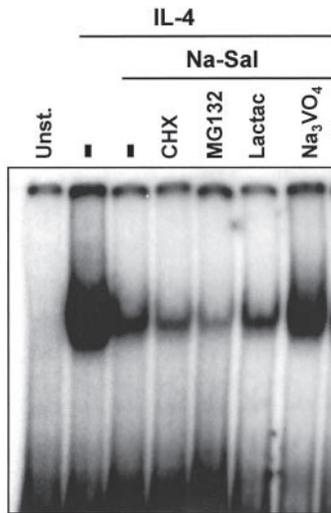


FIGURE 2. Phosphatase inhibitors partially block the effect of salicylates. M12 cells were stimulated with IL-4 for 10 min. Afterward, NaSal (20 mM) was added alone or together with cycloheximide (CHX; 20 μ M), MG132 (30 μ M), lactacystin (10 μ M), or Na_3VO_4 (1 mM) for 1 h. Afterward, STAT6 DNA-binding activity was analyzed by EMSA.

inhibited DNA-binding activity of STAT6 (Fig. 1B). Thus, a concentration of 20 mM salicylates completely blocked STAT6 activation, and a significant inhibition, greater than 50%, was still observed at 5 mM. In contrast to salicylates, treatment of cells with acetaminophen did not block STAT6 activation by IL-4 (Fig. 1C). High concentrations of acetaminophen (20 mM) did not abrogate STAT6 activation as compared with NaSal, and lower concentrations (10 and 5 mM) resulted in no inhibition. Cell viability was not affected under the experimental conditions used in these experiments, suggesting a specific effect of salicylates in inhibiting STAT6 activation.

Salicylates inhibit tyrosine phosphorylation by a JAK-independent pathway

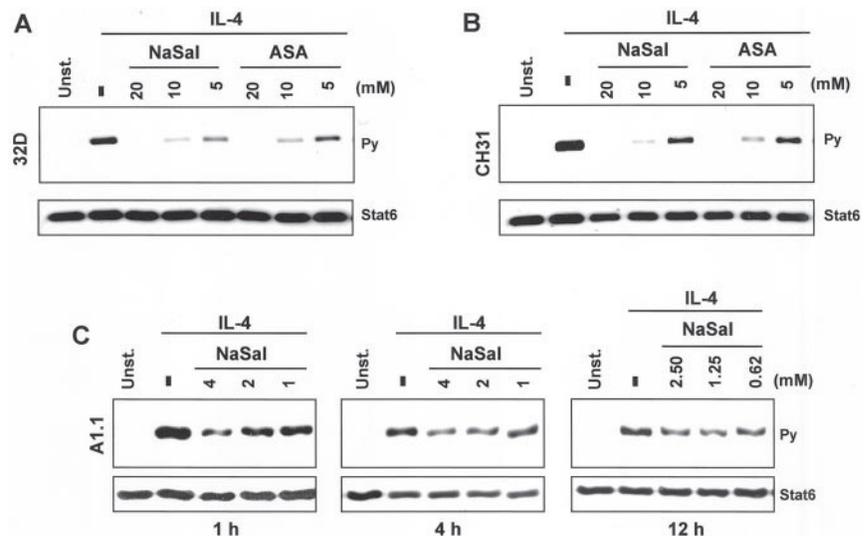
Protein kinases, phosphatases, and proteasome are involved in the regulation of STAT6 (36). To address the molecular mechanisms by which salicylates affected STAT6 activation, we analyzed the effect of proteasome and phosphatase inhibitors in this process (Fig. 2). We found that addition of salicylate after IL-4 stimulation

also resulted in the inhibition of STAT6. This inhibition was partially reverted by the phosphatase inhibitor Na_3VO_4 , but not by cycloheximide or proteasome inhibitors. Because Na_3VO_4 enhances the phosphorylation of STAT6 by IL-4 (37), likely through the inhibition of a constitutive phosphatase, our data suggests that kinases or phosphatases could be the target for NaSal because vanadate partially reverted its inhibitory effect.

Previous studies have established an effect of salicylates on kinase activation (18, 19). We focused on tyrosine kinases because serine phosphorylation of STAT6 is not required to bind DNA (38). Treatment of cells with salicylates resulted in the inhibition of tyrosine phosphorylation of STAT6 in the unrelated cell lines 32D, CH31, and A1.1 to the same extent they inhibited the ability of STAT6 to bind DNA (Fig. 3). In this case, NaSal and ASA completely abrogated the ability of IL-4 to promote STAT6 tyrosine phosphorylation at a concentration of 20 mM, and a substantial inhibition ($\sim 60\%$) was also present at 5 mM (Fig. 3, A and B). A significant inhibition was also observed at lower concentrations (<4 mM; Fig. 3C). Incubation of A1.1 cells with low doses of NaSal for 1, 4, and 12 h resulted in a reproducible inhibition of STAT6 phosphorylation. Similar results were obtained with 32D cells (data not shown). In these cases, densitometric scanning indicated that the levels of STAT6 phosphorylation are $\sim 50\%$ reduced in the presence of 4 mM NaSal and $\sim 30\%$ in the presence of 1 mM NaSal. Cell viability was not affected under these conditions of culture. Concentrations of salicylates in the millimolar range can be reached in plasma during treatment of rheumatic diseases and during analgesic and anti-pyretic regimens (10), suggesting that STAT6 activation may be inhibited during therapeutic treatment with salicylates.

We have shown that salicylates inhibit the activation of STAT6 in multiple cell lines. We next investigated the effect of salicylates on primary cells. Pretreatment of human PBMC with NaSal also inhibited the activation of STAT6 by IL-4 (Fig. 4A). The pattern of inhibition was similar to cell lines. Thus, 20 mM of NaSal completely block STAT6 activation and there was still a significant inhibition at low concentrations of 1–2 mM. We next investigated the physiological effects of STAT6 inhibition by salicylates. To this end, we analyzed the effect of salicylate treatment in the IL-4-induced CD23 expression on monocytic primary cells (Fig. 4B). We found that treatment of human PBMC with NaSal inhibited the induction of CD23 by IL-4 in a dose-dependent manner. Thus, 10 mM NaSal almost completely abrogated the induction of CD23,

FIGURE 3. Salicylates inhibit IL-4-induced STAT6 tyrosine phosphorylation. 32D (A) and CH31 (B) cells were incubated for 1 h with the indicated concentrations of NaSal and ASA, and were then stimulated with IL-4 for 30 min. C, A1.1 cells were cultured for 1, 4, and 12 h with the indicated concentrations of NaSal before IL-4 treatment. In all cases, STAT6 was precipitated in cell lysates using anti-STAT6 followed with protein G-agarose. Precipitates were separated by SDS-PAGE and immunoblotted with an antiphosphotyrosine Ab to detect tyrosine-phosphorylated (Py, upper blots). Membranes were stripped and re probed with anti-STAT6 Ab (Stat6, lower blots).



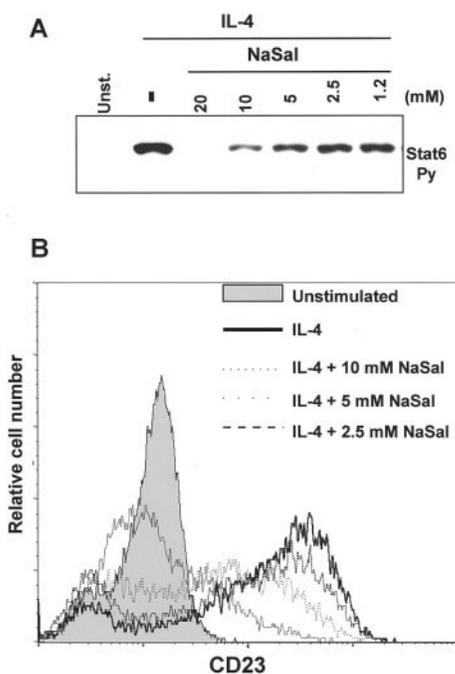
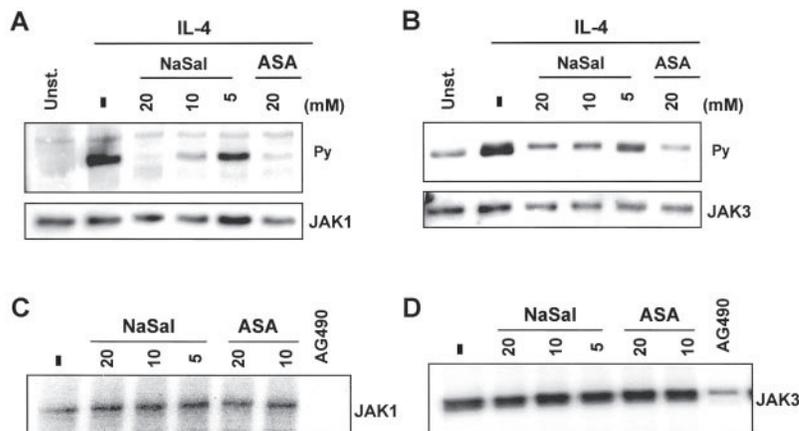


FIGURE 4. Salicylates inhibit IL-4-induced STAT6 tyrosine phosphorylation and CD23 expression on PBMC. *A*, Human PBMC cells were cultured with the indicated concentrations of NaSal for 1 h and were then stimulated with human IL-4 (10 ng/ml) for 30 min. Cell extracts equivalent to 0.4 millions of cells were separated by SDS-PAGE and immunoblotted with an Ab specific for phosphorylated STAT6. *B*, Human PBMC were cultured for 30 h in the presence of nothing (unstimulated), or IL-4 plus the indicated amount of NaSal. Afterward, cells were stained with FITC-conjugated anti-human CD23 Ab. The expression of CD23 was analyzed on viable monocytic cells by flow cytometry.

and a significant inhibition was still observed at low concentrations of 2.5 mM. Therefore, there was a correlation between STAT6 and CD23 inhibition. Similar inhibition of CD23 expression was obtained in the murine M12 cell line (A. D. Keegan, unpublished observations).

The tyrosine kinases JAK1 and JAK3 have been shown to play an important role in IL-4 signaling and activation of STAT6 (29, 30). Therefore, we tested the effects of salicylates on the IL-4-induced activation of these JAKs. Treatment of M12 cells with salicylates inhibited tyrosine phosphorylation of JAK1 and JAK3 to the same degree that they inhibited STAT6 phosphorylation (Fig. 5, *A* and *B*). However, salicylates had no effect on the kinase activity of these enzymes when analyzed in vitro (Fig. 5, *C* and *D*).

FIGURE 5. Salicylates inhibit in vivo, but not in vitro, JAK1 and JAK3. *A* and *B*, M12 cells were incubated with NaSal and ASA for 1 h before stimulation with IL-4 for 5 min. Cell lysates were treated with anti-JAK1 (*A*) or anti-JAK3 Abs (*B*) followed by protein G-agarose. Precipitates were separated by SDS-PAGE and were immunoblotted with an antiphosphotyrosine Ab to detect tyrosine-phosphorylated kinases (Py, upper blots). Membranes were stripped and reprobed with anti-JAK1 (*A*) or anti-JAK3 Abs (*B*; lower blots). *C* and *D*, M12 cell extracts were precipitated with anti-JAK1 (*C*) or anti-JAK3 (*D*). Precipitates were then incubated with nothing or the indicated amount of NaSal, ASA, or AG490 (50 μ M) for 20 min. The kinase activity of precipitates was then analyzed by autophosphorylation using radioactive ATP.



Concentrations of 20 mM, which completely blocked the activation of STAT6 and JAKs in treated cells, had no effect on the autophosphorylation of JAK kinases in vitro. In contrast, AG490, a known inhibitor of JAKs (39), blocked in vitro activity of JAK1 and JAK3. This data strongly suggested the possibility that another tyrosine kinase plays an important role in IL-4 signaling, and unlike the JAKs, this kinase would be directly affected by salicylates.

Salicylates inhibit an Src kinase involved in STAT6 activation

In addition to JAK kinases, several laboratories have demonstrated that the Src family of kinases can participate to varying degrees in the activation of STATs (40, 41). Although little is known about the role of Src kinases in IL-4 signaling, it has been shown that IL-4 stimulation can result in their activation (42). Interestingly, it has been reported that salicylates can inhibit the activation of Src kinases (43, 44). Therefore, we examined the IL-4-induced activation of Src family kinases and the effects of salicylates on this activation (Fig. 6). We found that IL-4 treatment of M12 cells activated an Src kinase that was precipitated by the anti-Src Ab, src2. We observed an increase in autophosphorylation activity in precipitates obtained from cells that had been stimulated with IL-4, and this increase was abrogated when cells were cultured in the presence of NaSal before IL-4 stimulation (Fig. 6*A*). Furthermore, the incubation of src2 immunoprecipitates with NaSal inhibited in vitro kinase activity (Fig. 6*B*). These results demonstrate that salicylates not only suppress the IL-4-induced activation of a Src kinase in cells, but also suppress the in vitro activity of this kinase.

To further confirm a role for Src in STAT6 activation, we analyzed the ability of IL-4 to signal STAT6 activation in cells expressing v-Src, a viral oncogenic form of Src (Fig. 7). NIH3T3 cells expressing v-Src showed a high basal tyrosine phosphorylation of STAT6 as compared with unstimulated wild-type cells (Fig. 7*A*). Stimulation of these cells with IL-4 also resulted in higher STAT6 phosphorylation than in control cells. As expected, treatment of parental NIH3T3 with NaSal inhibited the IL-4-induced phosphorylation of STAT6 (Fig. 7*B*). Salicylates also completely blocked the basal phosphorylation observed on v-Src-transformed cells. However, NIH3T3 cells expressing v-Src became more resistant to salicylate inhibition of IL-4-induced STAT6 phosphorylation. In this case, we observed a partial inhibition of phosphorylation on cells treated with 20 mM NaSal, but lower concentrations had no effect. This data suggests that the overexpression of src protects against salicylate inhibition, likely by augmenting the enzyme/drug ratio under these experimental conditions.

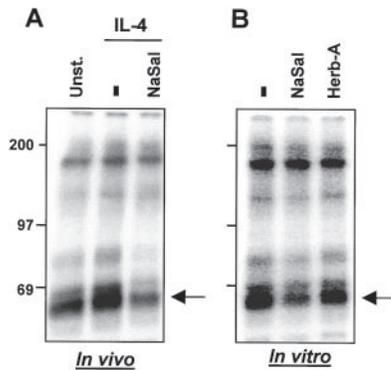


FIGURE 6. Salicylates inhibit an Src kinase involved in STAT6 activation. *A*, M12 cells were treated or not with 20 mM of NaSal before stimulation with IL-4. Cell extracts were incubated with src2, an anti-Src Ab, and were precipitated with protein G-agarose. The kinase activity of the precipitates was analyzed as in Fig. 5. *B*, Src2 precipitates were prepared from M12 cells and were incubated with nothing, NaSal (20 mM), or herbimycin A (10 μ M). Kinase activity was analyzed as above.

The role of Src in the IL-4-induced activation of STAT6 was also demonstrated in embryonic fibroblasts derived from Src-deficient mice (Ref. 34; Fig. 8). Treatment of the *src*^{-/-} cells with IL-4 stimulated a weak tyrosine phosphorylation of STAT6, whereas in wild-type cells, IL-4 treatment induced robust phosphorylation (Fig. 8A). In this case, NaSal treatment had similar inhibitory effect on wild-type and *src*^{-/-} cells (Fig. 8B). These data suggest that Src contributes to the IL-4-induced activation of STAT6, but other salicylate-sensitive kinases, probably members of the Src family, may substitute for this kinase.

Discussion

We have found in this study that salicylates, aspirin, and NaSal can inhibit STAT6 activation induced by IL-4 and IL-13, most likely by targeting a Src kinase. In contrast, acetaminophen (paracetamol) does not inhibit STAT6 activation. The effect of salicylates

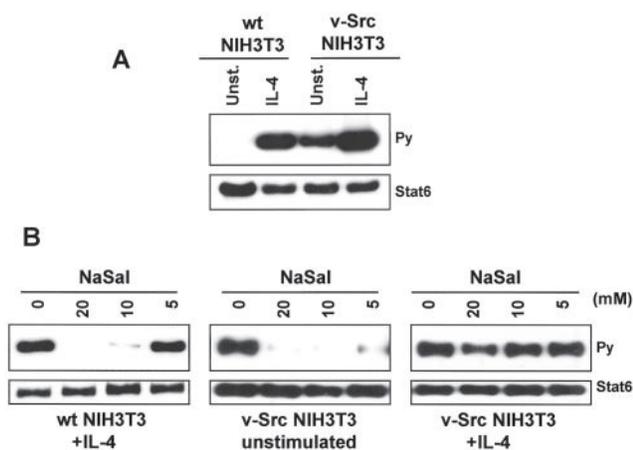


FIGURE 7. V-Src expression promotes STAT6 activation. *A*, STAT6 phosphorylation was analyzed in cell extracts obtained from wild-type or v-Src-expressing NIH3T3 cells stimulated or not with IL-4 (10 ng/ml). *B*, STAT6 phosphorylation was analyzed in wild-type NIH3T3 cells pretreated with NaSal before IL-4 stimulation (*left*), v-Src NIH3T3 cells cultured with NaSal without cytokine treatment (*center*), and v-Src NIH3T3 pretreated with NaSal and then stimulated with IL-4 (*right*). Culture conditions are the same as in Fig. 3A. The time of exposure in *B* was not the same in each sample. *Upper panels*, antiphosphotyrosine blots (Py). *Lower panels*, same membrane blotted with anti-STAT6 Ab (Stat6).

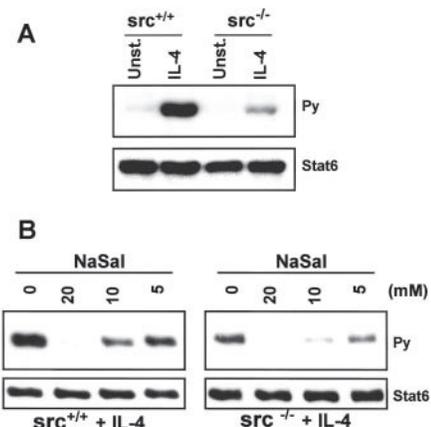


FIGURE 8. IL-4 induced low levels of STAT6 phosphorylation in cells lacking Src. *A*, STAT6 phosphorylation was analyzed as in Fig. 3 in embryonic fibroblasts derived from wild-type mice (*src*^{+/+}) or *src* knockout mice (*src*^{-/-}). *B*, STAT6 phosphorylation was analyzed in *src*^{+/+} (*left*) and *src*^{-/-} (*right*) cell lines pretreated with NaSal for 1 h with the indicated amount of NaSal before IL-4 stimulation for 30 min. *Upper panels*, antiphosphotyrosine blots (Py). *Lower panels*, same membrane blotted with anti-STAT6 Ab (Stat6).

on STAT6 activation correlates with the inhibition of CD23 expression, supporting a physiological role of salicylates in inhibiting IL-4 signaling.

A number of observations indicate that the effects of salicylates in IL-4 signaling are specific. First, cell viability was not affected under the experimental conditions used. Second, concentrations of salicylates lower than 5 mM, which can be achieved therapeutically and are not toxic (10), exert a significant inhibition of STAT6. Third, other authors have found, under similar experimental conditions, the opposite effect of salicylate on IFN- γ signaling. They have found that salicylates enhanced STAT1 activation (45).

We have found that an Src kinase is a target for salicylates in IL-4 signaling. COX enzymes have been thought to be the main target for aspirin and other nonsteroidal anti-inflammatory drugs. However, over the last years, a number of new targets of salicylates have been described, including p38 mitogen-activated protein kinase (18) and I κ B kinase complex (19). Our findings confirm previous reports showing the ability of salicylates to inhibit Src *in vivo* (43) and *in vitro* (44). Given the multiple functions associated with Src, it is reasonable that the effect of salicylates on Src could have important clinical applications.

Our study corroborates the evidence for a role of Src kinases in STAT activation (40, 41). Src kinases have been involved in the activation of several STATs by JAK-dependent (40) and -independent pathways (41). We have clearly shown that Src plays a critical role in the activation of STAT6. Furthermore, the fact that the absence or inhibition of Src abrogates JAK1, JAK3, and STAT6 activation suggests that Src activation is a very early event in IL-4 signaling. This effect on STAT6 is in contrast to a report showing the enhancement of STAT1 activation by aspirin (45), suggesting a divergence in the regulation of STAT1 and STAT6 by IFN- γ and IL-4, respectively. It is possible that this divergent effect could promote Th1-type responses while suppressing the Th2 type, with the result of inhibiting allergic disease.

Clinical studies have found a beneficial effect of salicylates and aspirin in allergic diseases, especially asthma (12–16). It has also been proposed that the substitution of aspirin by acetaminophen could have contributed to the increased incidence of asthma in children (5). This is in apparent contradiction with the fact that aspirin can actually precipitate asthma in a subset of asthmatic

patients (AIA) (17, 46). However, the effect of aspirin in these patients seems to be mediated by its direct effect on COX enzymes and its promotion of production of leukotriene C₄, not by an immunological response against aspirin (17, 47). Perhaps, paradoxically, salicylates have been used successfully to treat AIA patients. Treatment of patients with high doses of salicylates has been shown to offer a moderate protection against AIA in predisposed individuals (15, 16). These beneficial effects of salicylates in asthma treatment cannot be explained by their ability to block COX enzymes. In these studies, the doses used are higher than those required to block COX. In addition, NaSal that does not inhibit COX also has a beneficial effect on asthma. Therefore, the molecular mechanisms involved in these processes likely do not require COX regulation.

Given the importance of STAT6 and IL-4 in the induction of asthma (23–26, 31–33, 48), our data suggest that the beneficial effect reported for aspirin and salicylates in asthma may be mediated by the inhibition of STAT6 activation and thereby by a Th2-type immune response. Concentrations of salicylate lower than 5 mM that can be achieved during analgesic, antipyretic, and anti-inflammatory treatments are able to partially inhibit the activation of STAT6. This effect correlates with the ameliorating effects of salicylates on asthma. In addition, salicylic compounds such as gentisic acid, gallic acid, and 2,3-dihydroxybenzoic acid, which are products of aspirin degradation (10), can also inhibit Src kinase activity (44). Therefore, they may also potentially contribute to the inhibition of IL-4-induced STAT6 activation under physiological conditions.

The importance of IL-4 and IL-13 signaling in human asthma (48) and animal models of asthma (23–26, 31–33) has been established. Therefore, their signaling pathways may be good targets for therapeutic intervention of allergic diseases. The finding that salicylates are able to inhibit the signaling of these cytokines may lead to the design of novel treatments for these diseases.

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References

- Kay, A. B. 2001. Allergy and allergic diseases. *N. Engl. J. Med.* 344:30.
- Hopkin, J. M. 1997. Mechanisms of enhanced prevalence of asthma and atopy in developed countries. *Curr. Opin. Immunol.* 9:788.
- Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* 275:77.
- Sporik, R., S. T. Holgate, T. A. Platts-Mills, and J. J. Cogswell. 1990. Exposure to house dust mite allergen (*Der p* 1) and the development of asthma in childhood: a prospective study. *N. Engl. J. Med.* 323:502.
- Varner, A. E., W. W. Busse, and R. F. Lemanske. 1998. Hypothesis: decreased use of pediatric aspirin has contributed to the increasing prevalence of childhood asthma. *Ann. Allergy Asthma Immunol.* 81:347.
- Martínez, F. D., A. L. Wright, L. M. Taussig, C. J. Holberg, M. Halonen, and W. J. Morgan. 1995. Asthma and wheezing in the first six years of life. *N. Engl. J. Med.* 332:133.
- Holt, P. G. 1995. Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. *Pediatr. Allergy Immunol.* 6:1.
- Von Mutius, E., D. L. Sherrill, C. Fritzsche, F. D. Martínez, and M. D. Lebowitz. 1995. Air pollution and upper respiratory symptoms in children from East Germany. *Eur. Respir. J.* 8:723.
- Ball, T. M., J. A. Castro-Rodriguez, K. A. Griffith, C. J. Holberg, F. D. Martínez, and A. L. Wright. 2000. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *N. Engl. J. Med.* 343:538.
- Flower, R. J., S. Moncada, and J. R. Vane. 1985. Analgesic-antipyretics and anti-inflammatory agents: drugs employed in the treatment of gout. In *Pharmacological Basis of Therapeutics*, A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds. Macmillan, New York, p. 674.
- Committee on Infectious Diseases. 1982. Aspirin and Reyes Syndrome. *Pediatrics* 69:810.
- Szczeklik, A., and E. Nizankowska. 1983. Asthma improved by aspirin-like drugs. *Br. J. Dis. Chest* 77:153.

- Crimi, N., R. Polosa, S. Magri, G. Prosperini, G. Paolino, C. Mastruzzo, and A. Mistretta. 1996. Inhaled lysine acetylsalicylate (L-ASA) attenuates histamine-induced bronchoconstriction in asthma. *Allergy* 51:157.
- Sestine, P., R. M. Refini, M. G. Pieroni, A. Vaghi, M. Ribuschi, and S. Bianco. 1999. Different effects of inhaled aspirin-like drugs on allergen-induced early and late asthmatic responses. *Am. J. Respir. Crit. Care Med.* 159:1228.
- Nizankowska, E., R. Dworski, J. Soja, and A. Szczeklik. 1990. Salicylate pretreatment attenuates intensity of bronchial and nasal symptoms precipitated by aspirin in aspirin-intolerant patients. *Clin. Exp. Allergy* 20:647.
- Stevenson, D. D., M. A. Hankammer, D. A. Mathison, S. C. Christiansen, and R. A. Simon. 1996. Aspirin desensitization treatment of aspirin-sensitive patients with rhinosinusitis-asthma: long-term outcomes. *J. Allergy Clin. Immunol.* 98:751.
- Szczeklik, A., and D. D. Stevenson. 1999. Aspirin-induced asthma: advances in pathogenesis and management. *J. Allergy Clin. Immunol.* 104:5.
- Schwenger, P., D. Alpert, E. Y. Skolnik, and J. Vilcek. 1998. Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced I κ B α phosphorylation and degradation. *Mol. Cell. Biol.* 18:78.
- Yin, M. J., Y. Yamamoto, and R. B. Gaynor. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I κ B kinase- β . *Nature* 396:77.
- Kopp, E., and S. Ghosh. 1994. Inhibition of NF- κ B by sodium salicylate and aspirin. *Science* 265:956.
- Cianferoni, A., J. T. Schroeder, J. Kim, J. W. Schmidt, L. M. Lichtenstein, S. N. Georas, V. Casolaro. 2001. Selective inhibition of interleukin-4 gene expression in human T cells by aspirin. *Blood* 97:1742.
- Urban, J. F., N. Noben-Trauth, D. D. Donaldson, K. B. Madden, S. C. Morris, M. Collins, and F. D. Finkelman. 1998. IL-13, IL-4R α , and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *N. brasiliensis*. *Immunity* 8:255.
- Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186:1737.
- Grunewald, S. M., A. Werthmann, B. Schnarr, C. E. Klein, E. B. Brocker, M. Mohrs, F. Brombacher, W. Sebald, and A. Duschl. 1998. An antagonistic IL-4 mutant prevent type I allergy in the mouse: inhibition of the IL-4–13 receptor system completely abrogates humoral immune response to allergen and development of allergic symptoms in vivo. *J. Immunol.* 160:4004.
- Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183:109.
- Henderson, W. R., Jr., E. Y. Chi, and C. R. Maliszewski. 2000. Soluble IL-4R inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J. Immunol.* 164:1086.
- Russell, S. M., A. D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M. C. Friedmann, A. Miyajima, R. K. Puri, W. E. Paul, and W. J. Leonard. 1993. Interleukin-2 receptor γ -chain: a functional component of the interleukin-4 receptor. *Science* 262:1880.
- Miloux, B., P. Laurent, O. Bonnin, J. Lupker, D. Caput, N. Vita, and P. Ferrara. 1997. Cloning of the human IL-13R α 1 chain and reconstitution with the IL4R α of a functional IL-4/IL-13 receptor complex. *FEBS Lett.* 401:163.
- Chen, X. H., B. K. Patel, L. M. Wang, M. Frankel, N. Ellmore, R. A. Flavell, W. J. LaRochelle, and J. H. Pierce. 1997. Jak1 expression is required for mediating interleukin-4-induced tyrosine phosphorylation of insulin receptor substrate and Stat6 signaling molecules. *J. Biol. Chem.* 272:6556.
- Oakes, S. A., F. Candotti, J. A. Johnston, Y. Q. Chen, J. J. Ryan, N. Taylor, X. Liu, L. Henighausen, L. D. Notarangelo, W. E. Paul, et al. 1996. Signaling via IL-2 and IL-4 in JAK3-deficient severe combined immunodeficiency lymphocytes: JAK3-dependent and independent pathways. *Immunity* 5:605.
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Nebe, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258.
- Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261.
- Kuperman, D., B. Schofield, M. Wills-Karp, and M. J. Grusby. 1998. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.* 187:939.
- Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 64:693.
- Hershey, G. K., M. F. Friedrich, L. A. Esswein, M. L. Thomas, and T. A. Chatila. 1997. The association of atopy with a gain-of-function mutation in the α subunit of the interleukin-4 receptor. *N. Engl. J. Med.* 337:1720.
- Wang, D., R. Moriggl, D. Stravopodis, N. Carpino, J. C. Marine, S. Teglund, J. Feng, and J. N. Ihle. 2000. A small amphipathic α -helical region is required for transcriptional activities and proteasome-dependent turnover of the tyrosine-phosphorylated STAT5. *EMBO J.* 19:392.
- Huang, H., and W. E. Paul. 2000. Protein tyrosine phosphatase activity is required for IL-4 induction of IL-4R α -chain. *J. Immunol.* 164:1211.
- Pesu, M., K. Takaluoma, S. Aittomaki, A. Lagerstedt, K. Saksela, P. E. Kovanen, and O. Silvennoinen. 2000. Interleukin-4-induced transcriptional activation by STAT6 involves multiple serine/threonine kinase pathways and serine phosphorylation of STAT6. *Blood* 95:494.

39. Nielsen, M., K. Kaltoft, M. Nordahl, C. Ropke, C. Geirler, T. Mustelin, P. Dobson, A. Svejgaard, and N. Odum. 1997. Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. *Proc. Natl. Acad. Sci. USA* 94:6764.
40. Yu, C. L., R. Jove, and S. J. Burakoff. 1997. Constitutive activation of the Janus kinase-STAT pathway in T lymphoma overexpressing the Lck protein tyrosine kinase. *J. Immunol.* 159:5206.
41. Chaturvedi, P., M. V. Reddy, and E. P. Reddy. 1998. Src kinases and not JAKs activate STATs during IL-3 induced myeloid cell proliferation. *Oncogene* 16:1749.
42. Ikizawa, K., K. Kajiwara, T. Koshio, and Y. Yanagihara. 1994. Possible role of tyrosine kinase activity in interleukin 4-induced expression of germ-line C ϵ transcripts in a human Burkett lymphoma B-cell line, DND39. *J. Allergy Clin. Immunol.* 94:620.
43. Wang, Z., and P. Brecher. 2001. Salicylate inhibits phosphorylation of the non-receptor tyrosine kinases, proline-rich tyrosine kinase 2 and c-Src. *Hypertension* 37:148.
44. Ramdas, L., and R. A. Budde. 1998. The instability of polyhydroxylated aromatic protein tyrosine kinase inhibitors in the presence of manganese. *Cancer Biochem. Biophys.* 16:375.
45. Chen, L.-C., D. Kepka-Lenhart, T. M. Wright, and M. Morris. 1999. Salicylate-enhanced activation of transcription factors induced by interferon- γ . *Biochem. J.* 342:503.
46. Babu, K. S., and S. S. Salvi. 2000. Aspirin and asthma. *Chest* 118:1470.
47. Sanak, M., M. Pierzchalska, S. Bazan-Socha, and A. Szczeklik. 2000. Enhanced expression of the leukotriene C₄ synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. *Am. J. Respir. Cell Mol. Biol.* 23:290.
48. Borish, L. C., H. S. Nelson, J. Corren, G. Bensch, W. W. Busse, J. B. Whitmore, J. M. Agosti, and the IL-4R Asthma Study Group. 2001. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J. Allergy Clin. Immunol.* 107:963.